

## A preliminary disease survey in the wild Nile crocodile (*Crocodylus niloticus*) population in the Okavango Delta, Botswana

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### ABSTRACT

The objective of this study was to conduct a preliminary survey of diseases that might be present in the wild Nile crocodile population in the Okavango Delta, Botswana. Blood samples were collected from crocodiles ranging in size from 34.0 cm to 463.0 cm total length. Samples were examined for blood parasites and underwent a haematological analysis. Before release the crocodiles were examined for various clinical abnormalities. Of the 144 crocodiles examined, none were visibly sick or displayed any signs of disease. No antibodies to *Mycoplasma crocodyli* were detected. *Hepatozoon pettiti* was present in 55.3 % of blood smears examined, but there was no significant difference in any of the haematological values between the infected and uninfected crocodiles, and a high prevalence of *Hepatozoon* infection is not uncommon in other species. Only 7.6 % of the examined crocodiles were infested with leeches. Further research is required for several of the crocodilian diseases, in particular to elucidate the role of wild crocodilians as reservoirs of infection.

**Keywords:** Nile crocodile, *Crocodylus niloticus*, Okavango Delta, disease survey.

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### MATERIALS AND METHODS

#### Study site

Botswana's Okavango Delta, the world's biggest Ramsar site (a wetland of international importance), is a large wetland within the Kalahari Desert, covering an area of approximately 16 000 km<sup>2</sup> in the dry season and increasing to over 22 000 km<sup>2</sup> with the annual flood. The 111 250 km<sup>2</sup> active catchment area falls entirely within Angola. Owing to the geology of the catchment, the incoming water is low in nutrients and sediment<sup>32</sup>.

The Okavango River flows through Namibia briefly before entering Botswana and forming a broad floodplain, the Panhandle. An estimated 40 % of incoming water leaks into the surrounding swamps by the time the river leaves the Panhandle. The remaining 60 % is distributed down 3 main channels, which fan out to form the Delta. The Okavango Delta consists of permanent and seasonal swamp, which is inundated during the annual flood<sup>32</sup>.

The northern part of the Delta is characterised by shallow water, flooded grasslands, ox-bow lakes and lagoons mostly interconnected by narrow waterways. Only a few main channels lined by tall reeds (mainly *Phragmites australis*) carry the remainder of the Okavango's water southwards through the Delta. The permanent and seasonal swamp together form a unique ecosystem and provide a high-quality habitat for a great many species. As a keystone species, the Nile crocodile, helps maintain the fragile balance within this ecosystem. This includes selective predation on various fish species,<sup>10,42</sup> recycling of nutrients and maintenance of wet refugia in times of drought<sup>48</sup>. Crocodiles are unevenly distributed throughout the Delta with the majority of the breeding population occurring in the 120 km long Panhandle<sup>17</sup>.

#### Study animal

The Nile crocodile, *Crocodylus niloticus*, is the most widespread and abundant of the 3 crocodile species that occur in Africa. It occurs throughout the continent south of the Sahara in a variety of wetland habi-

### INTRODUCTION

Since the development of the crocodile farming industry in the 1980s, a number of studies have been carried out worldwide on diseases in farmed crocodiles<sup>6,7,12,13,20,27,28</sup>. Very little, however, is known about the diseases of wild crocodilians, particularly wild Nile crocodiles. Wild crocodiles are often difficult to study owing to the remoteness of the areas in which they occur.

The initial objective of this study was to determine disease prevalence in the wild Nile crocodile population in the Okavango Delta. However, several factors made this difficult:

- (i) Very few serological tests have been developed for crocodile diseases. Consequently a serological survey is of very limited value.
- (ii) Virus isolation is unavailable due to the lack of crocodile cell lines in veterinary diagnostic laboratories.
- (iii) Sick crocodiles are difficult to identify by observation. Under farmed conditions they often stop eating but show

no other clinical signs and therefore are not noticed to be unhealthy until they are found dead. Diseases causing visible external lesions, for example pox virus infection, are the obvious exception.

- (iv) Clinical examination has several limitations: cardiac and respiratory rates are variable according to conditions, and are strongly influenced by capture stress. Because crocodiles are poikilothermic, body temperature cannot be used as a diagnostic tool.
- (v) Sick/weak crocodiles are unlikely to survive for long due to predation. Therefore, the likelihood of encountering these individuals in a capture and release survey is low.
- (vi) Owing to research permit limitations no crocodiles could be sacrificed for histopathological and parasitological examination.

In view of these limitations, it was postulated that a low incidence of diseases would be found in the Okavango population during a capture and release survey. However, it was possible to do a more accurate survey for mycoplasmosis and haemogregarine infection thanks to the availability of diagnostic tests. In addition, ectoparasites, in the form of leeches, were easy to find, remove and count per individual.

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tats, including coastal areas<sup>41,46</sup>. Historically its distribution in southern Africa extended as far south as the Eastern Cape Province during the past 100–200 years<sup>42</sup>.

Nile crocodiles are ectothermic and regulate their body temperature behaviourally by moving between sun-exposed sandbanks and the water. Typical adult lengths are around 3.5 m, but the males can grow up to 5 m<sup>18</sup>. Sexual maturity is reached from 2.9 m total length for males, and 2.2 m for females<sup>10</sup>. Nesting occurs in a hole in the ground and on average 50 eggs are laid. Nile crocodiles exhibit temperature-dependent sex determination<sup>31</sup>. Hatchlings emerge after an incubation period of approximately 90 days in early to mid summer, and parental protection occurs<sup>39,40,41</sup>. As with other crocodilian species, a high mortality rate is experienced in their 1st year of life due primarily to predation<sup>45</sup>.

#### Capture methods

One hundred and forty-four crocodiles were captured in the Panhandle of the Okavango during summer (February 2005). Capture was carried out using 2 methods. At night, using a 4.8 m flat-bottomed aluminium motor boat, crocodiles were located with a spotlight which revealed the eyes glowing red. The beam of light was then kept focused on the crocodile's eyes, making it possible to approach the animal by boat. Crocodiles estimated to be smaller than 1.2 m total length were captured by hand. Crocodiles between 1.2 m and 2.3 m were captured using a swivelling noose (Animal Handling Co., SA) which was placed over the snout and pulled tight in the neck region. Crocodiles were then brought onto the boat, jaws were taped shut and the animals were physically restrained. Animals larger than 2.3 m were captured using a noose attached to a climbing rope, which was secured to the boat. The crocodile was allowed to swim so as to tire it out before it was brought onto the boat. In addition to night capture, baited box and Pitman traps were strategically placed on river banks. Traps were baited at sunset and checked at 1st light the next morning. Captured animals were immediately restrained, measured, examined and samples collected.

#### Crocodile processing

Each crocodile was blindfolded and restrained in ventral recumbency. Fifty-three animals were randomly selected for blood collection. Blood was collected from the post-occipital sinus<sup>9</sup> on the dorsal midline and just caudal to the base of the head. A 21 G (0.80 × 25–90 mm) or 23 G (0.65 × 25 mm) needle and a 3, 5 or 10 ml

syringe was used, depending on the size of the crocodile, and the blood was transferred directly into a lithium heparin tube. Blood smears were made from whole blood using the cover slip method<sup>24</sup>. Following blood collection, each crocodile was measured (total length (TL) and snout-to-vent length (SVL) using a flexible measuring tape, ±1 mm). It was then weighed using a harness placed around the forelimbs and a Pesola spring balance. Each crocodile was sexed by cloacal examination of the cliteropenis<sup>23,31</sup> and the entire body was examined for clinical abnormalities including bite wounds, skin lesions, conjunctivitis, joint swelling and poor condition. Dorsal and lateral body surfaces were also examined for the presence of leeches. Leeches were removed by means of a pair of tweezers, counted and stored in 70 % ethanol for later identification.

#### Sample processing

On return to the field laboratory 1.0 ml of blood was transferred to an Eppendorf tube for haematological analysis. The remaining blood was centrifuged using a manual desktop centrifuge and the plasma frozen for serology. If the volume of the blood sample was small it was allocated for either *Hepatozoon* examination or serology.

Thirty-eight samples were examined for blood parasites and underwent haematological analysis. Blood smears were stained with Diff-Quick Stain (American Scientific Products, Illinois, USA)<sup>8</sup>. The presence of *Hepatozoon* gametocytes was determined by microscopic examination of the Diff-Quick stained blood smears. A minimum of 3 slides per animal and in some cases more were examined including a minimum of 1000 HPF's (×100 oil immersion) per slide. The degree of erythrocyte regeneration was estimated by examining the red cell series and scoring each slide on a scale of 1 to 4.

On this scale, a score of 2 represented normal erythrocyte regeneration with 10–20 % orthochromic erythroblasts but no earlier stages. A score of 2.5 represented a moderate increase in erythrocyte regeneration, with late polychromatic erythroblasts through to mature and aging erythrocytes, while a score of 3 represented strong regeneration with basophilic erythroblasts and early polychromatic erythroblasts through to mature and aging erythrocytes, but no proerythroblasts. Finally, a score of 4 included all the other stages plus proerythroblasts.

Packed cell volumes (PCV) were determined using a Statspin MP microhaematocrit centrifuge: blood was drawn

into standard microhaematocrit tubes and centrifuged for 5 min at 12 000 g.

Total red cell counts (RCC) were performed both manually and automatically using an electronic particle counter. The automated counts were made using a Beckman Coulter Ac\*T Series haematology analyser (Coulter SA). The manual counts were made using Natt and Herrick's solution. A 1:200 dilution was made by drawing blood up to the 0.5 mark on a red blood cell diluting pipette, then filling the pipette to the 101 mark with Natt and Herrick's solution<sup>9</sup>. The diluted blood was then used to charge both counting chambers of an improved Neubauer haemocytometer (Hawksley and Sons, Lancing, UK). After 5 mins in a humidity chamber the red cells were counted in the 4 corner cells and central cell of the central large square of the counting chamber. This was repeated on the second chamber and the average multiplied by 10 000 to obtain the total red cell count per microlitre.

Haemoglobin concentrations (Hb) were determined using a Beckman Coulter Ac\*T Series haematology analyser (Coulter SA).

Red blood cell indices were calculated using standard equations<sup>24</sup>

Mean cell volume:

$$\text{MCV (fl)} = \text{PCV/RCC}$$

Mean cell haemoglobin:

$$\text{MCH (pg)} = \text{Hb (g/dl)} \times 10 / \text{RCC}$$

Mean cell haemoglobin concentration:

$$\text{MCHC (g/dl)} = \text{Hb (g/dl)} / \text{PCV}$$

Total white cell counts (WBC) were obtained indirectly using the Unopette 5877 system (Becton-Dickinson, USA). The Unopette pipette was filled with blood (25 µl) and mixed with the phloxine B diluent in the reservoir. From this, both counting chambers of an improved Neubauer haemocytometer were charged. After 5 min in a humidity chamber all the pink-staining granulocytes were counted in both chambers.

Differential counts were done on the Diff-Quick stained smears. The percentage of heterophils and eosinophils was calculated and used to calculate total WBC<sup>9</sup>.

Total WBC/µl =

$$\frac{\text{Stained cells counted in chambers} \times 1.1 \times 16 \times 100}{\text{Percentage heterophils and eosinophils}}$$

The separated plasma was stored at –10 °C in a domestic gas freezer for up to 1 month. After return from the research site, 30 samples were submitted to the laboratory (Faculty of Veterinary Science, Department of Veterinary Tropical Diseases, University of Pretoria) for serological

testing for mycoplasmosis, using a plate agglutination test.

#### Statistical analysis

Haematological values were analysed for significant differences ( $P < 0.05$ ) between *Hepatozoon*-infected and uninfected crocodiles by 1-way analysis of variance (ANOVA). The residuals were checked for normality of distribution with normal probability plots. Where data were not normally distributed, significance was tested by means of a Mann-Whitney test.

#### RESULTS

The crocodiles ranged in TL from 34.0 cm to 46.3 cm, with a mean of 39.7 cm, and ranged in SVL from 25.5 cm to 101.5 cm with a mean of 28.4 cm.

Of the 144 crocodiles caught and examined, none were visibly sick or displayed any clinical signs of disease. The body condition of all the crocodiles was good. The only external lesions observed were old healed bite injuries in one case, and a recent puncture injury in another case.

The only ectoparasite found on the Nile crocodiles examined was the leech *Placobdelloides multistriatus*. The leeches were identified with the aid of a key<sup>37,38</sup>. Eleven crocodiles were infested with the leech, a prevalence of 7.6 %. One crocodile had 7 leeches, and another had 2. The remaining 9 parasitised crocodiles each had a single leech. Leeches were found in various places on the crocodiles, both dorsally and ventrally, for example on the tail, neck, belly, armpits, between webbing of back legs, and other sites. There was no obvious pattern of leech distribution on the crocodiles. No correlation between current leech infestation and *H. pettiti* infection was found.

No antibodies to *Mycoplasma crocodyli* were detected in any of the sera tested.

*Hepatozoon pettiti* parasites were present in 21 of 38 blood smears examined (55.3 %). The mean SVL of the 38 crocodiles tested was 42.6 cm. The mean SVL of the infected crocodiles was 46.3 cm compared with 38.2 cm for the uninfected crocodiles. The youngest infected crocodile in this study had a SVL of 25.5 cm.

Six of 8 females (75 %) and 13 of 27 males (48 %) were infected. The sex of 2 crocodiles was undetermined due to their small size.

Two (9.5 %) of the infected crocodiles were infested with leeches, as were 4 (23.5 %) of crocodiles negative for *H. pettiti*.

Of the 21 infected crocodiles, 10 (47.6 %) showed an increased rate of red cell regeneration, with a mean score of 2.4 on the erythrocyte regeneration index. In

Table 1: Size and haematological parameters of *Hepatozoon*-infected and uninfected crocodiles.

Parameter	<i>Hepatozoon</i> infected (n = 21)		<i>Hepatozoon</i> negative (n = 17)	
	Mean	S.D.	Mean	S.D.
SVL (cm)	46.3	22.4	38.2	12.3
Mass (kg)	3.80	6.39	1.74	2.65
PCV Haematocrit %	18.2	2.0	17.6	1.9
RCCRBC ( $\times 10^6/\mu\text{l}$ )	0.62	0.14	0.56	0.08
Hb (g/dl)	7.27	1.11	6.90	0.82
MCV (fl)	305.4	70.6	320.7	46.0
MCH (pg)	121.5	31.8	125.2	13.7
MCHC (g/dl)	39.7	2.7	39.4	3.9
Eryth. regener. index	2.4	0.4	2.3	0.4
WBCWCC ( $\times 10^3/\mu\text{l}$ )	11.62	5.50	10.87	3.71
Heterophils %	19.8	8.7	21.5	8.9
Lymphocytes %	62.0	9.8	62.0	13.2
Monocytes %	1.0	2.2	0.6	1.1
Eosinophils %	6.0	5.1	3.5	4.1
Basophils %	5.7	4.4	6.2	4.7
Azuropils %	5.3	4.3	4.8	4.9
Heterophils ( $\times 10^3/\mu\text{l}$ )	2.00	0.62	2.19	0.89
Lymphocytes ( $\times 10^3/\mu\text{l}$ )	7.43	4.10	6.93	3.49
Monocytes ( $\times 10^3/\mu\text{l}$ )	0.11	0.22	0.07	0.11
Eosinophils ( $\times 10^3/\mu\text{l}$ )	0.65	0.58	0.38	0.51
Basophils ( $\times 10^3/\mu\text{l}$ )	0.70	0.75	0.66	0.54
Azuropils ( $\times 10^3/\mu\text{l}$ )	0.70	0.91	0.48	0.46

contrast, 6 of 17 (35.3 %) uninfected crocodiles displayed an increased rate of red cell regeneration, with a mean score of 2.3 on the erythrocyte regeneration index. This was not a significant difference.

There was no significant difference between any of the haematological values of the infected and uninfected crocodiles (Table 1). The mean PCV and RCC of the infected group were 18.2 % and  $0.62 \times 10^6/\mu\text{l}$ , respectively, compared with 17.6 % and  $0.56 \times 10^6/\mu\text{l}$  in the uninfected group.

#### DISCUSSION

Owing to the limitations mentioned in the introduction it is probable that a capture and release survey does not provide a conclusive reflection of disease prevalence in a crocodile population. On the other hand, a very low disease incidence in wild crocodiles is probable because stress is a very important predisposing factor to disease in crocodiles. Crocodilians have been found to respond to non-specific stress with a chronic increase in corticosterone secretion, inhibition of growth, inhibition of the reproductive system and suppression of the immune system<sup>30</sup>. Crocodiles living in a relatively pristine natural environment, with a low population density, will not be exposed to the stress experienced by farmed crocodiles in an artificial, intensive environment. Furthermore, transmission of infectious diseases under natural conditions is usually far slower due to less host-pathogen exposure compared with an intensive situation.

The 1st recorded outbreak of myco-

plasmosis in crocodiles occurred in Zimbabwe on 5 farms simultaneously<sup>34</sup>. Rearing stock 1–3 years of age developed swollen limb joints and lameness. Morbidity was 10 % and mortality even lower. A new species of *Mycoplasma* was cultured from the joints of affected animals and named *Mycoplasma crocodyli*<sup>26</sup>. The disease was then reproduced in healthy crocodiles by experimental infection with this isolate.

Also in 1995 a highly fatal outbreak of disease, characterised by arthritis and pneumonia, occurred in farmed alligators (*Alligator mississippiensis*) in Florida<sup>2</sup>. Nine out of 74 adult animals died over a 10-day period. A new species of *Mycoplasma* was isolated and later named *M. alligatoris*<sup>3</sup>. Losses continued over the next 6 months, despite tetracycline treatment, until only 14 alligators remained. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies produced by alligators in response to *M. alligatoris* exposure has since been developed<sup>4</sup>.

Following the initial outbreak in Zimbabwe, cases of mycoplasmosis were reported annually, from about 1/3 of Zimbabwean crocodile farms. An autogenous vaccine was developed which, in an experimental trial, afforded limited protection<sup>35</sup>. A subsequent severe outbreak occurred in crocodiles following translocation, in which the morbidity was over 50 % and the mortality rate over 20 %<sup>33</sup>. Subsequently mycoplasmosis has become an important disease on South African crocodile farms, with several severe outbreaks having occurred (J



Picard, University of Pretoria, pers. comm., 2006).

The epidemiology of this disease is not well understood. The source of the original Zimbabwean outbreak remains elusive. It was suggested that wild crocodiles may act as reservoirs of infection, with vertical transmission being the main mode of transmission<sup>35</sup>. However, unidentified mycoplasmas have been found in the faeces of farmed Nile crocodiles, indicating the possibility of horizontal transmission<sup>22</sup>. The concept of wild crocodilian reservoirs of infection has more recently been confirmed in the case of *M. alligatoris*. In a seroprevalence study, 5.4 % of wild American alligators were found to be positive for *M. alligatoris* antibodies, at 12 of 20 sites (60 %)<sup>5</sup>. Further elucidation of the epidemiology of mycoplasmosis in Nile crocodiles requires that the status of wild crocodiles be established.

Many crocodile farms in southern Africa (excluding South Africa) collect eggs from wild crocodile nests. If the wild population concerned is infected, this is a very risky practice. Often these farms are obliged to reintroduce a certain number of juvenile individuals into the wild. If, on the other hand, the wild population is free from mycoplasmosis, it could be disastrous to reintroduce infected juveniles.

At the time these samples were tested, the *M. crocodyli* plate agglutination test had just been developed. It was believed that the test could identify recently infected animals, but not old infections. Subsequent testing on a positive farm revealed that the test only identified very recent infection, as only animals that still had lesions tested positive (J Picard, pers. comm., 2006). The negative results obtained here do not, therefore, exclude historical infection with mycoplasmas. The *Mycoplasma* infection status of the Okavango crocodiles therefore remains uncertain. An indirect enzyme-linked immunosorbent assay (iELISA) test for *M. crocodyli* was recently developed<sup>11</sup>. This test has a high sensitivity and specificity (86 % and 100 %, respectively) and will be ideal for further testing of the Okavango population.

The Samochima crocodile farm in Botswana collects a maximum of 2000 eggs per nesting season from nests in the Panhandle region of the Okavango Delta. In return the farm is obliged to release 5 % of juvenile crocodiles back into the wild. Testing of these juveniles should be undertaken before release to avoid the risk of infecting the wild population.

Haemogregarines are protozoal blood parasites. Members of the genus *Hepatozoon* occur in various crocodilians. Asexual

schizonts are found in the liver of infected crocodiles and gametocytes are found in erythrocytes or free in the blood. Sexual multiplication occurs in the intermediate host, usually haematophagous insects and leeches<sup>25,29</sup>.

*Hepatozoon pettiti* was described in Nile crocodiles from Senegal and Uganda<sup>47</sup> and *H. sheppardi* was described from Nile crocodiles in Mozambique<sup>44</sup>. Hoare<sup>19</sup> was the first to show transmission of *H. pettiti* by the tsetse fly, *Glossina palpalis*. A later study<sup>14</sup> suggested that the leech *P. multilineata* was a vector of haemogregarines in alligators and this was supported by a further study a few years later<sup>15</sup>. Fairly recently it was confirmed that the species found in crocodiles in the Okavango is *H. pettiti*, although the vector is unknown at this stage<sup>16</sup>. *Hepatozoon* parasites are thought to be apathogenic in their crocodilian hosts. In this survey, the aim was to determine the prevalence of *H. pettiti* and its possible effect on the crocodile host.

In a study which ran concurrently to this one<sup>16</sup>, the authors found *H. pettiti* in 61 out of 186 Nile crocodiles (32.8 %). As in this study, no significant difference in the PCV of infected and uninfected groups was found. It is clear that there is a high prevalence of *H. pettiti* infection in the Okavango crocodile population, and that it appears not to be pathogenic to the crocodile host. The mean SVL of the *Hepatozoon*-infected crocodiles was greater than that of the uninfected group. It would appear that crocodiles do not lose the infection with increasing age.

A very high prevalence of *Hepatozoon* infection is not uncommon in other species. *Haemogregarina crocodilorum* was found to be widely distributed in the southern United States and was found in 77 (59 %) of 130 American alligators examined<sup>25</sup>. In another study<sup>1</sup> a *Haemogregarina* sp. was found in all of 9 wild alligators captured. They were anaemic compared with captive-bred controls. It is not clear whether the haemogregarine played a role in the anaemia, or whether it was caused solely by leech infestation and a lower nutritional plane of the wild alligators. A prevalence of 71.4 % was reported for *H. caimani* in caimans (*Caiman crocodilus*) in Western Brazil<sup>49</sup> and very recently a prevalence of 76 % was reported in *C. yacare* in Central Brazil<sup>50</sup>. A haemogregarine was found in 16 of 25 (64 %) salt- and fresh-water crocodiles (*C. porosus* and *C. novaeguineae*)<sup>28</sup>.

The ectoparasites of crocodilians have not been well studied and most reports to date refer primarily to ticks and leeches. However, only 7 publications have addressed tick parasitism of crocodili-

ans<sup>43</sup>. Leeches are a more common crocodilian ectoparasite and have been found on a few species including *C. porosus*<sup>52</sup>, *Alligator mississippiensis*<sup>14,15,25</sup>, *C. johnstoni*<sup>51</sup> and *C. niloticus*<sup>36,37</sup>. However, little is known about the possible pathogenicity of the various species, although leeches could possibly play a role in the transmission of crocodile-specific viral and bacterial infections<sup>21</sup> and as vectors of blood protozoans<sup>25</sup>.

*Placobdelloides multistriatus* has been recorded as a parasite on crocodiles in Africa<sup>10,36,37</sup> although there is no previous record of its occurrence on crocodiles in the Okavango region. A next step would be to determine whether *P. multistriatus* is a potential vector for *H. pettiti* infection in the Okavango's Nile crocodiles.

Further research is required on several of the crocodilian diseases to elucidate their epidemiology, and particularly the role of wild crocodilians as reservoirs of infection. Pox viruses, *Adenovirus*, West Nile virus, chlamydiosis and mycoplasmosis are all of particular interest.

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